

IMPROVED CARDIAC PERFORMANCE DURING REPERFUSION FOLLOWING
ISCHEMIA IN AGED RATS SUPPLEMENTED WITH VITAMIN E AND ALPHA-
LIPOIC ACID

BY

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Abstract of Dissertation Presented to the Graduate School
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The purpose of these experiments was to examine the effects of dietary antioxidant supplementation of vitamin E (VE) and alpha-lipoic acid (LA) on biochemical and physiological responses to *in vivo* myocardial ischemia and reperfusion (I-R) in aged rats. Male Fisher 334 rats (18 months old) were assigned to one of two dietary treatments: 1) control diet, or 2) VE and LA supplementation. The control diet, prepared to meet AIN-93M standards, contained 75 IU VE /kg diet. The antioxidant supplemented diet contained 10,000 IU VE /kg diet and 1.65 g LA/kg diet. Following a 14-week feeding period there was a significant increase ($p<0.05$) in myocardial VE levels in supplemented animals (mean values: control diet=39.1, supplemented=83.4 $\mu\text{g/g}$ wet wt). Following the feeding period, animals in each experimental group were subjected to an *in vivo* I-R protocol. Animals were anesthetized, mechanically ventilated, and coronary occlusion was achieved

by a ligature placed around the left coronary artery. Occlusion was maintained for 25 minutes followed by a 10-minute period of reperfusion. During the first five minutes of reperfusion, maximum systolic pressure was significantly higher ($p<0.05$) in antioxidant supplemented animals compared to control diet animals. Following reperfusion, hearts were removed and biochemical measurements were made and compared with hearts exposed to a sham surgery. I-R in control diet animals resulted in a significant increase ($p<0.05$) in two markers of lipid peroxidation compared to sham surgery control diet animals. However, in antioxidant supplemented animals exposed to I-R, there was a significant reduction ($p<0.05$) in lipid peroxidation compared to control diet I-R animals. In an effort to further understand the specific reactive oxygen species involved in the antioxidant-induced protection from lipid peroxidation, a series of experiments were conducted that oxidatively challenged heart homogenates from both control diet and supplemented sham surgery animals. The myocardium from antioxidant supplemented animals demonstrated significantly less oxidative damage when exposed to five different radical generating systems: 1) iron, 2) hydrogen peroxide, 3) hypoxanthine/xanthine oxidase, 4) 2,2'azobis(2-amidinopropane)-dihydrochloride, (AAPH), and 5) 2,2'azobis(2-4 dimethylvaleronitrile), (AMVN). These data indicate that supplementation with VE and LA protects the heart from lipid peroxidation and this protection is associated with improved contractile recovery during reperfusion following myocardial ischemia.

CHAPTER 1 INTRODUCTION

Cardiac injury resulting from ischemia-reperfusion (I-R) is a serious clinical problem with ischemia-induced pathological processes, such as heart disease, constituting the single greatest cause of death in this country {103}. Furthermore, myocardial I-R in the elderly results in greater myocardial dysfunction and slower recovery during reperfusion than in the younger heart {1, 93}. Therefore, there is great interest in interventions that would protect the aged myocardium against an ischemic insult and subsequent reperfusion. One such intervention that has shown cardioprotection in the young adult rat heart is prefeeding the animal with antioxidant compounds {28, 58, 59}.

The interest in antioxidant nutrition as a cardioprotectant results from findings that cytotoxic reactive oxygen species (ROS) are a major cause of I-R injury in the heart {18, 35, 44, 45, 75, 76, 90, 138}. ROS, including superoxide anions, hydroxyl radicals and peroxy radical formed during the early moments of reperfusion, induce myocardial lipid peroxidation and protein oxidation {16, 17, 84, 89}. We recently investigated cardioprotection against myocardial I-R-induced lipid peroxidation using the combination of two nutritional antioxidant compounds, vitamin E (VE) and alpha-lipoic acid (LA) {27}. Our results using *in vivo* I-R supported *in vitro* I-R findings {58, 59}, that a combination of these antioxidants provides protection against lipid peroxidation caused by myocardial I-R in the young adult rat heart. The effects of these compounds in aging animals remain unknown.

The main purpose of this study was to test the hypothesis that a combination of two dietary antioxidant compounds - VE and LA - will protect the aging myocardium from I-R damage. To test this hypothesis, rats were supplemented with these dietary compounds for

four months and then subjected to an *in vivo* myocardial I-R insult. Cardiac performance during I-R was monitored and biochemical assessment of oxidative damage on the heart was made following the surgery. Furthermore, the specific ROS which may be involved in an antioxidant-induced protection were examined. This was achieved by homogenizing heart samples from the supplemented and control diet animals and then subjecting them to various *in vitro* oxidative challenges. This facilitated a comparison of the relative abilities of each myocardium to quench different ROS and provided useful information in determining the ability of the antioxidant supplemented hearts to defend against different ROS. The rationale for this experimental approach is discussed in the forthcoming hypothesis justification section.

Significance

Heart disease is the most prevalent chronic disease in the United States, accounting for approximately 1.5 million heart attacks per year [103]. Therefore, protective strategies that result in a myocardium that is better able to resist an ischemic insult are clearly desirable. One such intervention is prefeeding with various antioxidant supplements, some of which have been shown to protect against ROS damage from I-R in young and adult animals [9, 38, 41, 42, 46, 52, 73, 74, 85, 91, 101]. The mechanisms by which nutritional antioxidants provide myocardial protection are not well understood, and disagreement exists as to which antioxidants are most effective in providing myocardial protection. Also, surprisingly little is known about the effects of antioxidant supplementation on the aged myocardium. Therefore, additional research that improves our understanding of the role of dietary antioxidants in providing protection from I-R injury is clearly warranted, especially in the aged heart which is more susceptible to I-R damage [1, 93].

Specific Aims

Dietary supplementation with VE and LA has been shown to provide protection against myocardial I-R damage in young adult rats [28, 58, 59]. This study was designed to determine if this intervention also protects the aging rat heart from I-R, and to determine which ROS are being protected against by this antioxidant combination. The following questions were addressed.

Primary Question

Will dietary supplementation with the combination of VE and LA reduce I-R mediated cardiac injury in aging animals?

Primary Hypothesis

Aging animals that consume supplementary VE and LA will experience less I-R-induced myocardial damage than animals consuming a control diet. This will be evidenced by decreases in markers of lipid peroxidation and protein oxidation.

Secondary Question

Compared to control animals, will heart homogenates from aging animals fed the antioxidant diet experience lower levels of lipid peroxidation when exposed to a) superoxide radicals, b) hydroxyl radicals, c) hydrogen peroxide and d) peroxy radicals generated by *in vitro* ROS challenge systems?

Secondary Hypothesis

Heart homogenates from aging animals fed the antioxidant diet will demonstrate lower levels of lipid peroxidation compared to control animals following exposure to superoxide radicals, hydroxyl radicals, hydrogen peroxide and peroxy radicals generated by *in vitro* ROS challenge systems.

Hypothesis Justification

We have previously demonstrated a reduction in I-R induced lipid peroxidation of the myocardium in VE and LA supplemented young adult rats {28}. At present, the efficacy of this treatment in elderly animals is unknown and the mechanisms responsible for protection remain to be elucidated. In the present study, the primary hypothesis is that the combination of these two interventions will also provide protection against I-R induced myocardial damage in aging rats. The justification for this hypothesis is as follows.

It has been demonstrated that the aged myocardium is more susceptible to biochemical damage and dysfunction during I-R than the myocardium of younger animals {1, 79, 93}. Aging also has been reported to result in a reduction in myocardial VE content {64} and an increased dietary requirement for this nutrient {5}. It is known that increasing the myocardial content of VE in hearts of young adult rats decreases the risk of tissue injury during oxidative stress {9, 38, 41, 42, 46, 52, 73, 74, 85, 91, 101}. In addition, it has been shown that the reduced form of LA (dihydrolipoic acid - DHLA), as well as being a potent antioxidant itself, also has the ability to recycle VE from its oxidized form {68, 70, 109}. Therefore, supplementary LA, most of which becomes reduced to DHLA inside cells, has the potential to further increase the VE concentration in the myocardium and increase the antioxidant status of the cardiac cell. Hence, it is speculated that supplementing aging animals with the combination of VE and LA, will provide cardioprotection against I-R injury due to the individual and synergistic antioxidant functions of these nutrients.

To assess myocardial damage, two measurements of lipid peroxidation and one measurement of protein oxidation were used. The hypothesis that this dietary combination will be associated with a decrease in both myocardial lipid peroxidation and cardiac protein damage is based on the known ROS quenching abilities of each antioxidant and the findings that both lipid peroxidation and cardiac protein oxidation occur during I-R {26,

55, 60, 106, 112]. It is speculated that protection from lipid peroxidation will be mainly due to the increased myocardial VE because it resides in the lipid phase of membranes and previous work in younger animals has shown similar findings {9, 38, 41, 42, 46, 52, 73, 74, 85, 91, 101}. In addition, DHLA has also been shown to inhibit lipid peroxidation {10,109}. Furthermore, LA and DHLA are found primarily in the cytosol {97} which permits an association with the cytoskeletal and contractile cardiac proteins susceptible to oxidation during I-R {120}. It is, therefore, speculated that increasing the LA and DHLA content of the aging myocardium will decrease the deleterious effects of cardiac protein damage.

To determine the specific ROS which are quenched in the myocardium of animals supplemented with VE and LA animals, heart homogenates from animals on both the control diet and the supplemented diet were subjected to *in vitro* oxidative challenges. The second hypothesis is that this antioxidant combination will protect against ROS generated by five different systems. This hypothesis is based on two different groups of studies. Firstly, research that has shown heart homogenates from VE fed animals protect against peroxy radicals generated *in vitro* by two of the systems used in this present study {80, 86}. Secondly, studies that have used either LA, its reduced form (DHLA) or their metabolites and have shown protection against ROS induced tissue damage generated by the other three systems {33, 34, 54, 105, 108, 110}. Therefore, we speculate that using a combination of these two antioxidants will protect against all five ROS generating systems. This will be demonstrated by less damage occurring in heart homogenates from the supplemented animals compared to control diet animals. To our knowledge, this is the first study to identify the specific ROS that are quenched by the myocardium of animals fed this antioxidant combination.

CHAPTER 2

REVIEW OF RELATED LITERATURE

Myocardial Ischemia-Reperfusion (I-R) Injury

Myocardial ischemia is a disease process characterized by reduced coronary blood flow, such that the supply of nutritive blood to the heart muscle is insufficient for normal cardiac aerobic metabolism. Prompt re-establishment of coronary flow by invasive and non-invasive clinical procedures is the most direct and effective means of limiting myocardial damage due to ischemia, although reperfusion carries with it an injury component. This reperfusion damage may reflect the toxic effects of reactive oxygen species (ROS) and their participation in degenerative cellular processes such as membrane lipid peroxidation and protein oxidation {26, 55, 60, 106, 112}. Although myocardial ischemia is a well-recognized clinical complication of atherosclerotic coronary disease, other processes such as coronary surgery or pharmacologically-increased coronary reperfusion also have the potential to cause I-R damage {64}.

Aging presents even further complications, with temporary ischemia such as angina frequently experienced by elderly individuals. Indeed, it has been shown that the aging heart is more susceptible to I-R damage than the younger heart {1, 93}. The high prevalence of coronary artery disease in the elderly has focused attention on strategies that may protect the aged myocardium from damage due to coronary occlusion and reperfusion. To design an effective strategy, it is first necessary to gain an understanding of the cellular mechanisms involved with myocardial ischemia and reperfusion that lead to impaired pump function and ventricular infarction.

The precise mechanisms contributing to cardiomyocyte I-R injury are multi factorial and not yet completely understood [8]. At present, ROS are considered to be one of the primary pathogenic agents in cardiac I-R [115, 138]. Other mechanisms that have also been shown to promote myocardial damage include calcium overload due to impaired ATP production [8], increased activation of proteases and lipases due to elevated calcium [126], and platelet/leukocyte aggregation and activation resulting in further coronary occlusion as well as the release of coronary vasoconstrictors such as thromboxane [78].

The myocardial dysfunction which occurs during post-ischemia reperfusion is believed to be caused by damage to the sarcoplasmic reticulum resulting in dissipation of the important transsarcolemmal calcium gradient and an increase in cytosolic calcium concentration [43]. This leads to a sustained contractile activation resulting in hypercontracture, distortion of the myocardial cytoskeleton and diminished contractile performance [122]. The extent of contractile dysfunction is dependent on the period of ischemia with greater than five minutes of ischemia resulting in myocardial stunning and a subsequent decreased contractile performance. Upon reoxygenation of hypoxic heart tissue, contractile performance generally remains depressed for several days. The damage to the sarcoplasmic reticulum is believed to be due, at least in part, to an increased production of ROS during reoxygenation of the hypoxic tissue. ROS have been shown to participate in degenerative cellular processes such as membrane lipid peroxidation and protein oxidation [26, 55, 60, 106, 112]. Damage to the sarcoplasmic reticulum membrane and calcium transport proteins by ROS may result in an increase in cytosolic calcium concentration leading to myocardial dysfunction. The present study hypothesizes that this chain of deteriorating might be attenuated by pre-loading the myocardium with nutritional antioxidants that decrease the ROS-induced damage to the sarcoplasmic reticulum membrane.

Reactive Oxygen Species (ROS) and I-R Injury

Sources of ROS

ROS such as the peroxy radical (ROO'), the superoxide anion ($\text{O}_2^{-\cdot}$), the hydroxyl radical (OH'), and hydrogen peroxide (H_2O_2) are derived from the univalent reduction of molecular oxygen (56). Certain ROS are known as free radicals because they contain an unpaired electron in their outer orbital (e.g. ROO' , $\text{O}_2^{-\cdot}$, OH'). When a ground state O_2 molecule accepts a single electron, the product is $\text{O}_2^{-\cdot}$ and addition of a second electron yields H_2O_2 . Homolytic fission of the O-O bond in H_2O_2 produces two OH' and can be achieved by a simple mixture of H_2O_2 and an iron salt. This reaction is called the Fenton reaction, and the OH' produced react at extremely high rate constants with almost every type of molecule found in living cells {81} making them the most cytotoxic of the ROS.

Potential sources of ROS during I-R are the mitochondrial respiratory chain, xanthine oxidase activity, enzymatic arachadonic acid oxygenation, nitric oxide synthesis, catecholamine oxidation and the neutrophil oxidative burst {64}. Another implicated mechanism is the reductive stress associated with oxygen deprivation during ischemia {48, 99}. Of these, the enzymatic oxidation by xanthine oxidase of purines such as xanthine and hypoxanthine is believed to be one of the major sources of ROS during I-R in rodents {24, 131}. Mainly located in the vessel walls of most tissues, including cardiac muscle, the enzyme xanthine dehydrogenase (XDH) catalyzes the oxidation of hypoxanthine to xanthine, and xanthine to uric acid. During I-R, XDH may either reversibly or irreversibly be transformed to xanthine oxidase (131). In contrast to its dehydrogenase form, xanthine oxidase utilizes O_2 as the electron acceptor and produces $\text{O}_2^{-\cdot}$ while catalyzing the oxidation of hypoxanthine to uric acid {61}. In the post-ischemic myocardium, activated neutrophils

are also known to release large amounts of ROS that have also been associated with I-R injury {115}. The relative contribution of any of these sources of ROS in the pathogenesis of myocardial dysfunction remains to be defined.

The cardiotoxicity of ROS is largely reflective of their ready participation in processes that result in cellular dysfunction {55}. These processes include lipid peroxidation, protein oxidation and DNA damage {115}. While many biological molecules may be targets for oxidative stress, it is clear that during myocardial I-R cardiac proteins and the cell membrane lipids are particularly vulnerable {26, 53, 55, 60, 106, 107, 120}.

Myocardial Lipid Peroxidation

Multiple unsaturation points in polyunsaturated fatty acids (PUFA) make them highly susceptible to ROS attack and oxidative damage. Lipid peroxidation is the destruction of PUFA in membranes and is initiated when ROS have sufficient energy to abstract H⁺ atoms from methylene groups of the PUFA backbone forming ROO[·], which then can react with another PUFA, starting a chain reaction that amplifies the destructive effects {4}. ROO[·] are particularly dangerous because they are also capable of propagating further oxidative damage to other molecules such as proteins {27}. The major problem with lipid peroxidation within membranes is the alteration of the fluidity and permeability. Compromised cell membrane integrity has been suggested to be the most deteriorative reaction that occurs during I-R {127}, invariably leading to arrhythmias and cell death {115}. By-products of lipid peroxidation such as malondealdehyde, lipid hydroperoxides and conjugated dienes are measured to determine the extent of oxidative stress placed on a tissue {55}.

Cardiac Protein Oxidation

Oxidative damage to cardiac proteins by ROS includes destruction of contractile, cytoskeletal and membrane bound proteins and enzymes. Protein oxidation might involve polymerization, polypeptide chain scission, cross linking of proteins, and changes in individual amino acids {127}. Quantitative studies of protein oxidation show that proteins containing a sulfhydryl group are more susceptible to damage. An important protein which contains multiple sulfhydryl groups is the ryanadine receptor on the sarcoplasmic reticulum which is responsible for calcium handling. Protein oxidation of the ryanadine receptor might result in an increase in cytosolic calcium and myocardial dysfunction. Another group of proteins which have been reported to be susceptible to oxidation are the proteins of the contractile machinery, i.e., myosin and the thin filament complex {60}. Disruption of contractile proteins would also lead to muscle dysfunction. Furthermore, the cytoskeletal proteins such as vinculin and alpha-actinin that are responsible for the structural integrity of the myocyte have also been shown to be sensitive to I-R in both human and animal myocardium {60}.

In summary, the combined effects of protein oxidation and lipid peroxidation have a profoundly detrimental impact on the function of the cell {127}. However, the myocardium has intrinsic mechanisms designed to minimize oxidative damage, including both endogenous and exogenous antioxidants. The following sections will highlight key components of the myocardial antioxidant defense system.

Antioxidant Defense Mechanisms

Given the potential role of ROS in disease processes, it is not surprising that cells have extensive endogenous and exogenous protective systems to avoid damage from free radicals. Primary defenses include both enzymatic (superoxide dismutase, catalase, and

glutathione peroxidase) and non-enzymatic compounds including vitamins E, C and beta carotene, glutathione ,LA and its reduced form; dihydrolipoic acid (DHLA). These antioxidants work both separately and together attempting to maintain a reduced environment within the cell that will protect against oxidative damage.

Vitamin E

VE is considered to be an ideal naturally occurring antioxidant {97}. It is the most widely distributed antioxidant and is the primary chain-breaking molecule in cell membranes {21}. The generic term VE refers to at least eight structural isomers of tocopherol {15}. Among these, alpha-tocopherol is the best known and possesses the most potent antioxidant activity {21}. Protection of membranes by VE is dependent on the incorporation of alpha-tocopherol into membranes {51, 128}. Under most dietary conditions the concentration of VE in tissues is relatively low {64}. For example, VE levels in biological membranes is usually less than 0.1 nmol per mg of membrane protein or, in other words, one molecule per 1000-2000 membrane phospholipid molecules {97}. Because membrane levels of VE are low, numerous investigators have attempted to elevate tissue levels by either infusion or dietary supplementation.

Supplementation of Vitamin E

At high doses, the absorption of dietary VE decreases and more is excreted in the feces {82}. Studies of absorption in rats have shown that as the oral dose of radioactive alpha-tocopherol increases the percent absorption decreases from 70% at the recommended rodent intake (75 IU/kg diet) to 30% at the megadose level (20,000 IU/kg diet) {32}. However, once VE is absorbed there appears to be little metabolism in the circulation as demonstrated by only small changes in the excretory metabolites {83}.

Although it is difficult to saturate heart tissue with VE, myocardial membranes can accumulate VE during dietary supplementation, and concentrations in the rat heart appear to peak around fourteen weeks after the initiation of supplementation {28, 83}. The exact mechanisms responsible for the ability of tissue to increase VE concentrations over time are not known, but probably involves an increased uptake by the cell, improved retention and a decreased consumption by ROS{13, 83}. Furthermore, two cytosolic alpha-tocopherol binding proteins (TBP) have been identified that are capable of binding and transferring alpha-tocopherol to organelle membranes such as the sarcoplasmic reticulum {36, 92}. It has been postulated that an increase in the extracellular concentration of VE results in an increase in the TBP and a greater incorporation of alpha-tocopherol into membranes {128}.

VE has a relatively long biological half-life (2.4 days) in the rat {83} and has few, if any, side effects even when administered in megadoses {21, 28, 37, 71, 83, 133, 135}. The LD₅₀ of alpha-tocopherol for rats is >2 g/kg body weight {128}. This converts to > 30,000 IU/kg diet. Machlin and Gabriel {83} fed rats 10,000 IU/kg diet (1% of the diet) for 20 weeks and measured tissue and serum levels of tocopherol throughout this time period. The serum levels with this megadose were around 3 mg/dl, which they report are similar to values obtained when high levels of VE are given to humans, indicating the relevance of this high dose. In support of these findings, our laboratory recently completed a VE feeding study in which the same dose (10,000 IU/kg diet) was fed to rats for 14 weeks. We found similar myocardial VE levels after 14 weeks (increased by approximately 400%) and no signs of toxicity {28}.

Antioxidant Properties of Vitamin E

As an antioxidant, VE is particularly important because of its ability to convert ROO' to less reactive forms. VE can also break the lipid peroxidation chain that occurs during free

radical damage and is the major free radical chain terminator in membranes {133}. While VE is an efficient radical scavenger, the interaction of VE with a radical results in a reduction of functional VE and the formation of a VE radical. Oxidative stress has been shown to significantly reduce tissue VE levels {20, 64}. However, the VE radical can be "recycled" back to its native state by a variety of other antioxidants such as LA, vitamin C and glutathione {68, 70, 109}. It is postulated that the ability of VE to serve as an antioxidant is synergistically connected to other antioxidants that are capable of recycling VE during periods of oxidative stress {69}. This point is discussed in the section on LA.

Vitamin E Supplementation and I-R Injury

Because VE possesses many of the criteria considered to be a good antioxidant, numerous investigations have examined the effects of VE on I-R injury in the heart {9, 38, 41, 42, 46, 52, 73, 74, 85, 91, 101, 116}. Conceptually, infusion or dietary supplementation of VE could provide protection against I-R injury and experimental support for this notion is growing. Whole organ and animal models of myocardial ischemia, as well as ischemic heart disease patients, have been studied to investigate whether VE might be effective in preventing post-ischemic myocardial dysfunction/necrosis.

VE treatment of *in vitro* hypoxic rat hearts has been shown to be effective in preventing irreversible cardiomyocyte injury with reoxygenation, as well as reducing the contractility and mitochondrial derangements {9, 38, 41, 42, 46, 52, 74, 85, 91, 101}. In isolated heart models of no-flow (global) ischemia, VE has been shown to quench lipid radical formation during reperfusion {9, 41}. A 51% increase in the myocardial content of VE *in vivo* protects rat hearts against injury subsequently induced *in vitro* by 25 minutes of global ischemia {85}. The results of these isolated heart studies constitute compelling evidence

that cardiac VE protects against myocardial I-R injury *ex vivo*. The molecular mechanisms responsible for this protection remain to be determined.

Only recently have well controlled attempts been made to investigate if the benefits of VE observed *in vitro* in I-R hearts. Infusion of VE prior to 45 minutes of ischemia was reported to limit myocardial infarction in pigs, whereas the same dose of VE administered only during ischemia was less effective {73}. VE has also been shown to reduce ventricular fibrillation during chronic ischemia in the rat {42}. These limited studies suggest that VE is effective as a cardioprotectant against the irreversible injury associated with I-R episodes.

However, not all investigations on the effects of dietary VE supplementation on myocardial I-R have yielded positive outcomes {73, 116}. Although the reasons for these discrepant findings are unclear, at least three potential explanations exist. First, one study that failed to show that VE protected against I-R injury used pigs as the experimental model {73}. However, dietary supplementation of VE does not appear to increase VE levels in the porcine heart {73}. Hence, dietary VE supplementation in pigs would not be expected to provide protection against an I-R insult.

A second reason for the differential findings with VE supplementation may be the differences in experimental design. For example, experimental differences in VE dosage, the type of I-R protocol (i.e., *in vitro* vs. *in vivo*), and/or the duration of I-R may contribute to the lack of agreement in the literature. It has been argued that *in vivo* I-R models are more physiologically applicable {64}. Additionally, the dose and duration of VE supplementation are both critical for increasing tissue levels of the vitamin. Although the most common feeding period is four weeks, this results in only small increases in VE storage (e.g., ~30%) even when large doses are given {83}. In a study by Shuter et al. {116}, manipulation of myocardial alpha-tocopherol levels failed to affect functional recovery following an ischemic challenge in the rat heart; the feeding period, however, was 4 weeks and myocardial VE content was only increased by 40%.

A final possible explanation for the failure of some studies to show that VE supplementation provides protection against I-R injury may be because radical scavenging consumes VE leaving the antioxidant prematurely exhausted. Indeed, numerous investigators have argued that cellular antioxidant systems are most effective when two or more antioxidants work together {22, 64, 97, 137}.

Vitamin E and Aging

A new and important observation is that VE levels in the heart decline with age {64}. Although there is no consensus in the literature regarding this finding, it implies that there may be an increased dietary need for VE during senescence. In support of this notion, Ames {5} studied the effect of age on VE requirement in rats and found that the requirement increased markedly with age. The compounded rates of requirement were 7.6%/week from 11 to 43 weeks of age and 12.5%/week from 43 to 59 weeks of age. Furthermore, Chen et al. {25} reported that older mice required more dietary VE than younger mice to prevent lipid peroxidation in liver homogenates.

Several possibilities could explain why there is an increased dietary requirement for VE with aging. First, intestinal absorption of VE could decrease with aging. However, Hollander and Dadufalxa {63} reported an age-related increase in the total amount of VE absorbed; these investigators concluded that this was an adaptation designed to increase the systemic availability of VE with aging. A second possibility is that VE is metabolized in the circulation prior to uptake by tissues. Nonetheless, this does not appear to occur as Machlin and Gabriel {83} reported no change in the urinary concentration of the major metabolite of alpha-tocopherol. A third possibility is that there is a decreased tissue uptake of the vitamin. Although the exact mechanism of VE uptake has not been elucidated it is believed to occur by passive diffusion {15}. It follows that the rate of diffusion is dependent upon the VE concentration gradient between the plasma and the lipid phase of

the cell membrane as well as the availability of lipid carriers {15}. However in experiments using isolated myocardial cells, it was reported that the uptake of VE does not change with aging {50}. A final possibility is that once inside the aged cell VE is called upon to quench more ROS than in younger animals, thus increasing the requirement. In support of this hypothesis, many investigators have shown a decrease in antioxidant enzyme activity in aged animals {23, 30, 113, 118}. This would increase the burden on other antioxidants such as VE and may help explain why the requirement of VE changes with age.

In humans, aging has been shown to alter nutrient intake, increase the need for specific nutrients, and interfere with the storage and utilization of nutrients {117}. Persons older than 60 years are prone to disorders that lead to vitamin deficiencies. In addition, decreases in functional capability and ability to masticate might result in a decrease in total nutrient intake. Combined, these findings justify a supplementary intake of antioxidant vitamins.

Alpha-lipoic Acid

The antioxidant supplement LA is presently receiving a great deal of interest from the scientific oxidative stress community. LA is an endogenous thiol which serves as a cofactor for multienzyme complexes that catalyze the oxidative decarboxylation of alpha-keto acids such as pyruvate and alpha-ketoglutarate {97, 121}. LA was tentatively classified as a vitamin after its isolation but was later found to be synthesized by animals and humans {119}. It is known by a variety of names including lipoic acid and thioctic acid. Although the *in vivo* metabolism of exogenous LA has not been completely elucidated, it is believed that after entering the cell it is reduced to dihydrolipoic acid (DHLA) {97}. Furthermore, both LA and DHLA undergo beta oxidation to shorter chain metabolites such as bisnorlipoate, tetrnorlipoate and beta-hydroxybisnorlipoic acid (69, 72, 87, 88, 97, 98, 119). At physiological pH the salts of LA dissociate and lipoate is

formed. Therefore, determination of LA levels in physiological fluids or tissues is specifically a measure of lipoate content.

Antioxidant Properties of Alpha-lipoic Acid, Dihydrolipoic Acid and their Metabolites

Normally, lipoate is present in very small quantities (1-5 nmol/g) in animal tissues and is generally bound to an enzyme complex, rendering it unavailable to serve as an antioxidant [97]. The antioxidant benefits obtained from consuming supplementary LA may be due to at least five possibilities. Firstly, unbound lipoate is an effective antioxidant against a wide variety of ROS including O_2^- , OH⁻ and H₂O₂{14, 49, 97, 123, 125}. Secondly, the reduced form of LA (DHLA) has also been reported to be a potent antioxidant against the major oxyradical species {69, 97, 98, 125}. In addition, DHLA has been shown to be an important agent in recycling vitamin C, which in turn recycles VE during periods of oxidative stress. A fourth explanation concerns the influence of supplementary LA on cellular glutathione levels. Lipoate has been reported to be an effective glutathione substitute {10, 21, 22} also capable of increasing cellular glutathione levels {59}. A fifth possibility is that shorter chain metabolites of both LA and DHLA also have antioxidant properties. Although it is not known what proportion of LA and DHLA is converted to these metabolites, it has been reported recently that reduced forms of these metabolites scavenge a number of ROS both in aqueous solutions and in liposomes {14, 124}. Indeed, the bimolecular kinetic rate constant for the superoxide reaction was higher for both bisnorlipoate and tetrano-norlipoate than for DHLA {124} indicating that these shorter chain analogs also possess potent antioxidant capability.

Alpha-lipoic Acid and I-R Injury

In a number of myocardial I-R model systems, LA or DHLA have been effective in preventing damage. *In vitro*, DHLA prevents I-R induced changes in fluidity and polarity of rat heart mitochondria {108}. Several studies extend these findings to more closely match *in vivo* situations {6, 58, 59, 114}. Rats fed LA were protected against I-R injury induced in an isolated perfused Langendorff heart with improved post-ischemic left ventricular functional recovery and decreased lipid peroxidation {114}. Assadnazari et al. {6}, using a working heart system in an NMR magnet, found that DHLA added to the reperfusion buffer accelerated the recovery of aortic flow during reperfusion. The mechanism of protection of lipoate is difficult to elucidate as in none of these studies were the cellular levels of lipoate measured. The observation that LA caused an increase in intracellular glutathione led investigators in a similar study to hypothesize that the increased glutathione was responsible for protection from the I-R mediated damage {58}.

Vitamin E and Alpha-lipoic Acid

Figure 1 illustrates the role of DHLA and vitamin C in the recycling of VE during periods of oxidative stress. The interaction of VE with a radical results in the formation of a VE radical (vit. E⁻). The VE radical can be recycled by vitamin C at the cost of forming a vitamin C radical (dehydroascorbate). This radical can be reduced back to vitamin C by DHLA; DHLA is then converted to LA in this process and can be reconverted to DHLA by cellular enzymatic mechanisms {69}.

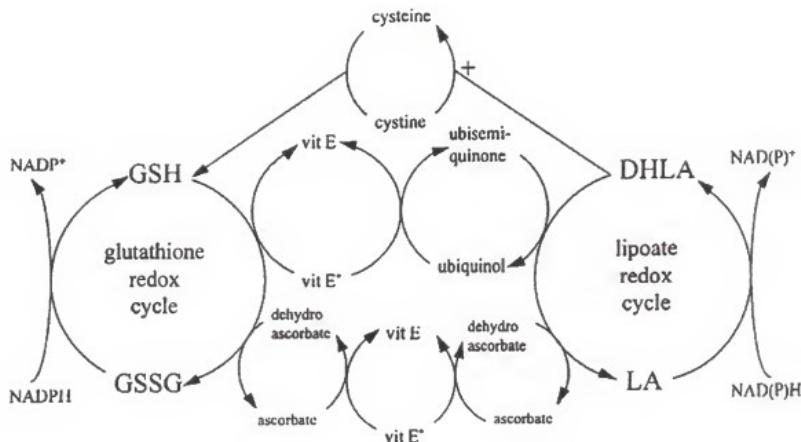


Figure 1. Vitamin E cycle

Thus, it appears that LA and DHLA act as antioxidants not only directly, through radical quenching, but indirectly as well, through recycling of other antioxidants [97]. Therefore, in theory, dietary supplementation of both VE and LA could also synergistically provide protection against oxyradical stress.

Vitamin E, Alpha-lipoic Acid and I-R Injury

Two published studies have used the combination of VE and DHLA (the reduced form of LA) to determine their effectiveness in preventing cardiac I-R injury [58, 59]. Both studies used the same dietary supplementation protocol for VE along with an *in vitro* assessment of I-R injury. In both investigations the DHLA was perfused in the buffer. In the first study, VE feeding increased myocardial VE content but did not show any improvement in functional recovery [59]. Control hearts perfused with DHLA also did not show any improvement. However, a synergistic response was observed with the combination of DHLA perfusion and high dietary VE using both the working heart system

and the Langendorff system. To elucidate the mechanism responsible, this research group carried out the same experiment and measured ATP levels [59]. They found that in hearts perfused with a buffer containing DHLA, ATP levels were significantly higher than those of control hearts but without improved functional recovery, which was consistent with the first experiment. In the VE supplemented animals, the myocardial content of reduced glutathione was significantly higher. In contrast, significantly higher levels of the oxidized form of glutathione (GSSG) were found in the DHLA perfused group without VE supplementation. This was thought to be due to membrane stabilization that stopped the GSSG from leaving the cell. They concluded that the mechanisms responsible for protection by VE and DHLA are different [59]. These findings suggest that the combination of VE and DHLA may be useful in providing tissue protection against I-R injury. However, to date, there are no *in vivo* reports examining this possibility; this formed the basis for the present experiments.

CHAPTER 3 METHODS AND PROCEDURES

Animals and Feeding Treatment

Seventy two Fisher-344 rats (18 months old) were divided into four dietary groups, two control and two antioxidant supplemented. The animals in the control groups were fed the AIN-93M purified diet, which contains 75 IU/kg diet dl-alpha-tocopherol acetate. The animals in the antioxidant supplemented group were fed the control diet along with 10,000 IU/kg diet dl-alpha-tocopherol acetate and 1.65 g/kg diet LA. The diets were professionally prepared by Harlan Teklad Inc. (Madison, WI).

After completing a fourteen week feeding protocol, animals in the four groups underwent the following surgical procedures;

- Group 1) Control diet - I-R surgery
- Group 2) Control diet - Sham surgery
- Group 3) Antioxidant supplemented diet - I-R surgery
- Group 4) Antioxidant supplemented diet - Sham surgery

Ischemia-Reperfusion Protocol

The animals in the I-R surgery groups underwent an *in vivo* I-R protocol. The sham animals underwent the same surgical interventions without I-R in order to provide baseline data for the myocardial levels of oxidative damage and antioxidants and to provide tissue for the heart homogenates that were subjected to *in vitro* oxidative challenges.

Animals in the I-R surgery groups were anesthetized with 50 mg/kg sodium pentobarbital and ventilated with room air. Rectal temperature was maintained at 37°C with a heating blanket. Electrical activity of the heart was monitored using a standard limb (lead II) ECG. Arterial pressure and heart rate were measured by inserting a catheter into the ascending aorta via the carotid artery. The chest was opened via a left thoracotomy and a ligature was placed around the left anterior descending coronary artery close to its origin. In the I-R surgery animals, coronary occlusion was achieved by passing both ends of the ligature through a small plastic tube which was then pressed on the surface of the heart directly above the coronary artery. The resulting arterial occlusion was maintained for 25 minutes by clamping the plastic tube and ligature with small hemostats. Reperfusion was achieved by removing the hemostats and the tube. Cardiac performance was monitored for 10 minutes during reperfusion. Hearts were then quickly removed, placed in an ice-cold antioxidant buffer to remove excess blood, then rapidly frozen in liquid nitrogen and stored at -80°C for subsequent biochemical analyses.

Animal Model Justification

The male Fisher-344 rat was chosen as an experimental model because: 1) the nature of these invasive experiments prevents the use of human subjects, 2) the Fisher-344 rat is highly inbred and does not display large inter-animal variation in coronary collateral circulation [11], 3) ischemia-reperfusion induced injuries in the rat and human are similar [31], 4) the Fisher-344 rat is a widely accepted model for the study of age-related myocardial adaptations [1, 105].

Validation of Coronary Occlusion and Reperfusion

The previously mentioned technique of coronary occlusion has been used successfully by our laboratory and others {17, 28, 65}. To validate that coronary occlusion was achieved in this model, we performed preliminary experiments where small amounts of Evans blue dye were injected directly into the right atrium (i.e. downstream from the ligature). After injection of the dye, the heart was removed within 10 seconds and examined for evidence of dye in the ventricular mass supplied by the left anterior descending coronary artery. Failure to observe dye stain in this area of the ventricle was interpreted as achievement of coronary occlusion.

To ensure that reperfusion had been adequately achieved in these preliminary experiments, we also administered Evans blue dye at the end of the 10 minutes reperfusion period. In each case we observed a uniformly stained heart; this was interpreted as evidence that reperfusion had occurred {31}.

Assessment of Myocardial Performance

To monitor myocardial performance arterial pressure was measured by placing a fluid-filled catheter into the ascending aorta via the left carotid artery using techniques described by Geenen et al. {47}. The catheter was connected to a pressure transducer and interfaced with a computerized heart performance analysis system (Digi-Med, Louisville, Kentucky). Cardiac function was determined prior to ischemia and constantly during the I-R protocol by measuring peak systolic pressure.

Assessment of Myocardial Antioxidant Status

Determination of Myocardial Vitamin E

Myocardial levels of VE were determined via high performance liquid chromatography (HPLC) using the protocol of Cort et al. {29}. Tissue preparation for VE analysis consisted of homogenizing 35 mg of the left ventricle in 2 ml acetone using a polytron homogenizer (Ultra-Turrax T25 , IKA Works, Cincinnati, Ohio); 2 ml of 10% pyrogallol in 100% ethanol was added and the samples heated for two minutes at 70°C; 300 µL of saturated KOH was then added followed by incubation for 30 minutes at 70°C. After cooling on ice, one ml of deionized water was added to each sample, followed by double extraction with HPLC-grade petroleum ether and centrifugation at 700 x g for five minutes. The petroleum ether phase was dried by evaporation under a stream of nitrogen and then reconstituted in isoctane (the HPLC mobile solvent) before injection into the HPLC. An ABI Analytical Spectroflow 400 HPLC was used for the VE analysis. Aliquots (20µL) of the isoctane extract were injected onto a 250 x 4 mm, 10µm LiChrosorb SI column (Baird and Tatlock, Dagenham, UK).

Determination of Myocardial Lipoate

At physiological pH, the salts of LA dissociate and lipoate is formed. Therefore, determination of LA levels in physiological fluids or tissues is specifically a measure of lipoate content. HPLC was used to determine myocardial lipoate content using a modification of the technique of Scholich et al. {109}. Frozen left ventricle samples were crushed to powder using a mortar and pestle while adding liquid nitrogen to prevent thawing. One ml of 20% metaphosphoric acid was added to the crushed tissue and this was homogenized using a teflon-head homogenizer. Three ml of HPLC-grade hexane and

0.25 ml of iso-propanol were added and sonicated in an ice water bath for 15 minutes and then shaken on ice for 10 min. After the samples were centrifuged at 200 x g for five minutes, the upper hexane layer was collected and the extraction procedure repeated twice. The pooled hexane extractant was evaporated under liquid nitrogen and reconstituted in ethanol for analysis. A microsorb (10 cm x 3 μ m C18 column) (Rainin Instruments, Massachusetts) was used with a water/methanol/acetonitrile mobile phase (50/30/20) and five g/L monochloroacetic acid.

Biochemical Assessment of Antioxidant Enzyme Activity

To determine if the dietary treatments altered myocardial antioxidant enzyme activity, a small sample of the left ventricle from all animals was assayed for the activities of total superoxide dismutase (SOD), manganese superoxide dismutase (mnSOD), copper-zinc superoxide dismutase (cu-znSOD), glutathione peroxidase (GPX), and catalase (CAT). The myocardium was minced and homogenized in cold 100 mM phosphate buffer w/ 0.05% bovine serum albumin (1:100 wt/vol; pH = 7.4). Homogenization with a polytron homogenizer was followed by centrifugation at 400 x g for 10 minutes. The supernatant was then removed and assayed for total SOD, mnSOD and cu-znSOD using the nitrite inhibition technique of Oyanagui {96}. Selenium GPX activity was determined using the procedure described by Flohe and Gunzler {39}. CAT was assayed using the protocol described by Aebi {3} and modified by Ji et al. {65}. Our coefficients of variation for GPX, and CAT and all SOD assays are ~five, three and four percent, respectively. These and other spectrophotometric biochemical analyses were performed in duplicate at 25°C, and samples from all experimental animals were assayed on the same day to avoid interassay variation.

Biochemical Indicators of Oxidative Stress

Two damaging processes that occur due to ROS are peroxidative damage to lipids and oxidation of proteins. To determine the extent of oxidative stress in the myocardium following I-R, two measures of lipid peroxidation and one measure of protein oxidation were used.

Lipid Peroxidation Measurements

To determine the amount of ROS-mediated oxidative damage in the heart, left ventricular levels of two by-products of lipid peroxidation were measured. Malondialdehyde levels were determined spectrophotometrically using the thiobarbituric acid-reactive substance (TBARS) method previously described by Uchiyama and Mihara {134} with 1,1,3,3-tetramethoxypropane used as the standard.

Lipid hydroperoxides were quantified using the ferrous oxidation/xylenol orange technique reported by Hermes-Lima {62}. Cumene hydroperoxide was used as the standard for this assay. The coefficient of variation for the TBARS and the lipid hydroperoxide assays are ~3 and 4 percent, respectively.

Protein Oxidation

To quantify the amount of protein oxidation that occurred during I-R, total protein carbonyl derivatives were measured spectrophotometrically as described by Reznick and Packer {104}, with modifications reported by Yan et al. {136}. Briefly, tissue protein was extracted in a protease-inhibitor (0.5 μ g/ml leupeptin, 0.7 μ g/ml antipain, 0.5 μ g/ml aprotinin, 40 μ g/ml phenylmethylsulfonylfluoride, one mM EDTA) treated 100 mM potassium phosphate buffer, pH 7.4. Nucleic acids were removed from samples with 1%

streptomycin sulfate treatment. The sample was then treated with one mM 2,4-dinitrophenylhydrazine (DNPH). The protein was then washed in ethyl acetate-ethanol (1:1 vol/vol) and dissolved in 6 M guanidine hydrochloride, pH 2.3. Tissue protein carbonyl content was quantified by scanning the samples from 350 to 390 nm in a spectrophotometer. The peak absorbance was used to calculate protein carbonyl content (extinction coefficient 22,000 l/mol/cm).

In Vitro Measurement of Tissue Antioxidant Capacity

To further define the antioxidant potential of VE and LA supplementation, heart homogenates from the sham surgery animals were subjected to five different ROS generating systems and then analyzed for lipid peroxidation using the TBARS assay. A section of the left ventricle from both supplemented and control animals was homogenized at a concentration of 10:1 in either 0.9% (w/v) saline solution (for the aqueous generating systems) or in ethanol (for the lipid phase system). Aliquots of the homogenates were incubated at a concentration of 10 mg protein/ml in the presence or absence of an ROS generating system according to the method of Haramaki et al. {59}. The following is brief descriptions of the five systems that were used.

Hypoxanthine-Xanthine Oxidase System

Superoxide radicals were generated by a hypoxanthine-xanthine oxidase system according to the method of Fridovich {40}. Briefly, one ml of one mM xanthine and 0.1 international units xanthine oxidase were added to a one ml aliquot of heart homogenate and incubated at 37°C for 120 minutes.

Hydrogen Peroxide System

One ml of hydrogen peroxide (100 µM) was added directly to one ml aliquots of heart homogenates and incubated at 37°C for 30 minutes according to the method of Scott {112}.

Hydroxyl Radical System

Hydroxyl radicals were generated in the heart homogenates by adding one ml of 0.1 µM ferric chloride (FeCl_3) to one ml aliquots of heart homogenates. The mixture was then incubated at 37°C for 15 minutes. The choice of this concentration was based on work by Bernier et al. {12} who found that this dose of iron elicited free-radical mediated arrhythmias in the isolated perfused rat heart and that it was possible to prevent iron induced arrhythmias by concomitantly perfusing the heart with SOD.

AAPH System

Peroxyl radicals were generated in the aqueous phase of the homogenate by thermal decomposition of 2,2'azobis(2-amidinopropane)-dihydrochloride, (AAPH). The rate of decomposition of AAPH is determined primarily by temperature, and to a minor extent, by solvent and pH {94}. At 37°C in neutral water, the half-life of AAPH is about 175 hours so the rate of free radical generation is virtually constant for the first few hours {130}. The AAPH was prepared by dissolving an appropriate amount of AAPH (final concentration of AAPH was 9.4 mM) {69} in an aqueous solution containing 0.9% (w/v) saline. One ml of AAPH solution and one ml of heart homogenate were then mixed and incubated at 37°C for 90 minutes.

AMVN System

Peroxyl radicals were generated in lipids in the heart homogenate by thermal decomposition of 2,2'azobis(2-4 dimethylvaleronitrile), (AMVN). The AMVN was prepared in ethanol at a concentration of five mM {69}. The same quantity (one ml) and temperature (37°C) was used for the AMVN solution and the mixture was incubated for 30 minutes.

Following incubation of the heart homogenates in each system, butylated hydroxytoluene BHT (200mM) and deferioxamine (One mM) in ice cold trichloroacetic acid (20% w/w) were added to stop the oxidative reaction. TBARS formation was then analyzed as previously described.

Statistical Analysis

Assessment of myocardial performance and myocardial levels of vitamin E and lipoate were subjected to a students t test. All other biochemical parameters were subjected to a one-way analysis of variance with a Scheffe test used post-hoc. Significance was established at p<0.05.

CHAPTER 4 RESULTS

Overview of Experimental Findings

These experiments examined the effects of dietary supplementation with antioxidants (VE and LA) on myocardial physiological and biochemical responses during *in vivo* ischemia-reperfusion (I-R) in the aging rat. The major findings of the study were that antioxidant supplementation resulted in improved cardiac contractile performance during reperfusion (figure 4) and a significant reduction in myocardial lipid peroxidation resulting from the I-R insult (figures 5 and 6). In an effort to determine the specific ROS involved in the antioxidant-induced protection, a series of experiments were conducted that oxidatively challenged heart homogenates from both control diet and supplemented sham surgery animals. Our data indicate that the myocardium from antioxidant supplemented animals sustained significantly less oxidative damage when exposed to a wide range of ROS generated *in vitro* (figures 8 and 9). Collectively, these data indicate that supplementation with VE and LA results in a heart which resists lipid peroxidation by a number of different reactive oxygen species and that this protection is associated with improved recovery following myocardial ischemia. Details of experimental findings are outlined in the following sections.

Myocardial Vitamin E Concentration

Figure 2 contains the mean (\pm SEM) myocardial VE concentrations in both control and supplemented diet animals. These data indicate that our feeding protocol resulted in

significant increase in the myocardial VE levels in the supplemented animals compared to animals consuming the control diet ($p<0.05$).

Myocardial Lipoate Concentration

Figure 3 contains the mean (\pm SEM) myocardial lipoate concentrations in both control and supplemented diet animals. No significant differences ($p=0.32$) in lipoate levels existed between the two groups.

Cardiac Contractile Function

Myocardial performance was assessed during ischemia and reperfusion utilizing a fluid filled catheter placed in the ascending aorta measuring peak systolic pressure. The effects of the different diets on aortic peak systolic pressure during the experimental protocol are shown in figure 4. Note that no significant differences existed between experimental groups prior to and during ischemia. However, peak systolic pressure was significantly ($p<0.05$) higher at two and five minutes during reperfusion in the antioxidant supplemented animals compared to control diet animals. At one and ten minutes of reperfusion, the peak systolic pressure was higher in the antioxidant supplemented animals compared to control diet animals, however the difference was not statistically significant; (at one minute, $p=0.14$, at ten minutes, $p=0.10$).

Lipid Peroxidation

Two markers of lipid peroxidation were used to determine the effects of the different diets on cardiac damage due to I-R. Figures 5 and 6 contain the mean (\pm SEM) values for

myocardial TBARS and cumene hydroperoxide equivalents (CHE) levels in both experimental groups. First, note that very similar results were obtained using the two markers of lipid peroxidation. Also notice that in the control diet group, I-R surgery resulted in a significant increase ($p>0.05$) in both myocardial TBARS and CHE levels compared to sham surgery animals. Finally, a key finding was that antioxidant supplemented I-R animals had significantly lower ($p<0.05$) TBARS and CHE levels compared to control I-R animals.

Myocardial Antioxidant Enzymes

Table I contains mean (\pm SEM) myocardial activities of glutathione peroxidase (GPX), total superoxide dismutase (SOD), manganese superoxide dismutase (mnSOD), copper-zinc superoxide dismutase (cu-znSOD) and catalase (CAT). Several observations are noteworthy. Firstly, compared to control diet animals, myocardial GPX activity in antioxidant supplemented animals was significantly higher ($p<0.05$) in both the sham and the I-R surgery groups. Secondly, SOD activity was greater ($p<0.05$) in supplemented animals who underwent I-R surgery compared to control diet I-R surgery animals. Further, control diet I-R surgery animals possessed lower ($p<0.05$) total SOD activity compared to control diet sham surgery animals. Analysis of the different isoforms of SOD indicated that these differences in total enzyme activity were due to changes in the manganese isoform of the enzyme. Finally, dietary supplementation with VE and LA did not alter the myocardial activity of CAT in either sham surgery or I-R surgery groups.

Protein Carbonyls

Protein carbonyls were assayed as a measure of protein oxidation in the myocardium of all experimental groups (figure 7). Myocardial carbonyl concentrations did not significantly differ ($p>0.05$) between any of the experimental groups.

In Vitro Oxidative Challenges

The myocardium from supplemented animals was significantly ($p<0.05$) better protected against lipid peroxidation in all four aqueous radical generating systems compared to the myocardium from control diet animals (figure 8). Similarly, compared to control, hearts from antioxidant supplemented animals experienced less ($p<0.05$) lipid peroxidation when exposed to the lipid phase (AMVN) oxidative challenge (figure 9).

Table 1. Summary of the effects of antioxidant supplementation and I-R surgery on endogenous antioxidant enzymes.

<u>Antioxidant Enzyme</u>	<u>Control diet</u>		<u>Antioxidant supplemented diet</u>	
	<u>Sham surgery</u>	<u>I-R surgery</u>	<u>Sham surgery</u>	<u>I-R surgery</u>
<i>n</i>	11	15	10	14
Glutathione Peroxidase ($\mu\text{mol}/\text{min}./\text{mg protein}$)	0.36 \pm 0.02	0.43 \pm 0.04	0.49 \pm 0.04**	0.58 \pm 0.03**
Total Superoxide Dismutase (Units/mg protein)	81.6 \pm 3.5	74.5 \pm 3.5*	78.8 \pm 3.1	80.2 \pm 2.8**
Cu-Zn Superoxide Dismutase (Units/mg protein)	36.9 \pm 2.2	42.3 \pm 4.4	36.2 \pm 2.0	36.8 \pm 2.9
Mn Superoxide Dismutase (Units/mg protein)	44.6 \pm 2.9*	32.2 \pm 2.2*	42.6 \pm 2.6	43.4 \pm 2.3**
Catalase (Units/mg protein)	1.50 \pm 0.07	1.68 \pm 0.12	1.57 \pm 0.07	1.64 \pm 0.06

Values are group means (\pm SEM).

n, sample size.

* different from sham surgery with same diet ($p<0.05$).

** different from control diet group with the same surgical treatment ($p<0.05$).

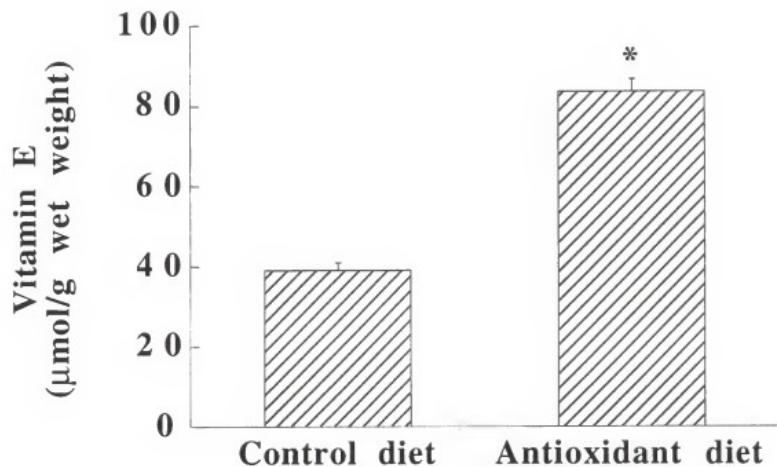


Figure 2. Comparison of myocardial vitamin E concentrations between control diet and supplemented diet animals.

Values are means (\pm SEM).

* significantly greater than control diet ($p < 0.05$).

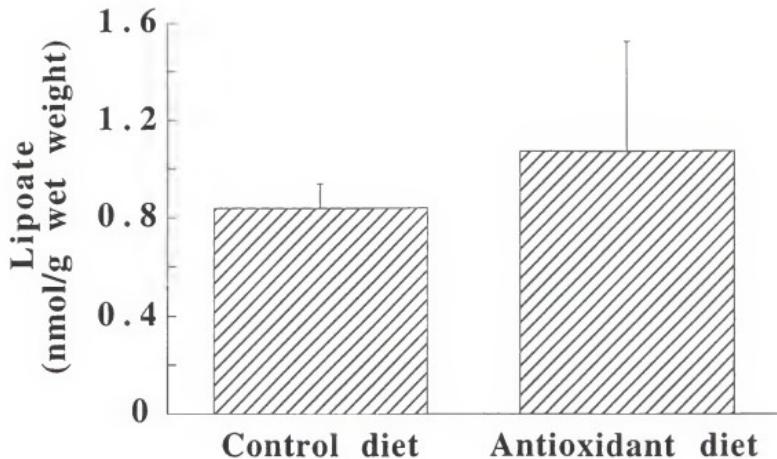


Figure 3. Comparison of myocardial lipoate concentrations between control diet and supplemented diet animals.

Values are means (\pm SEM).

No significant difference ($p=0.32$).

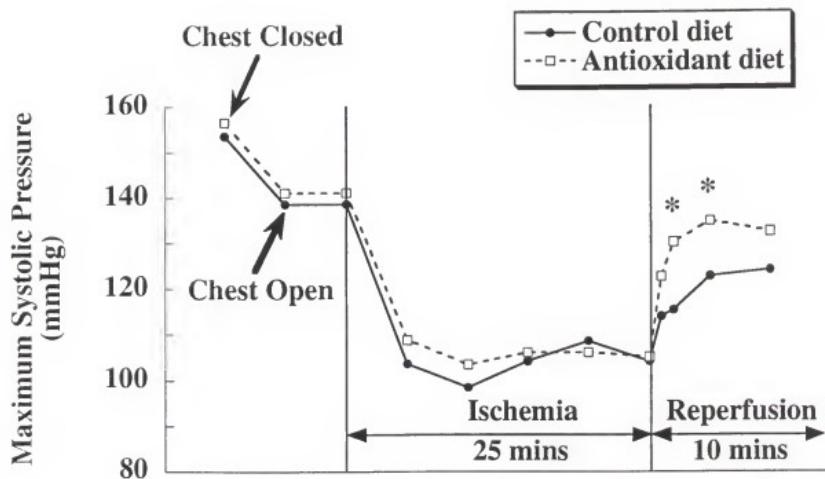


Figure 4. The effects of control and antioxidant diets on peak systolic pressure during ischemia and reperfusion.

Values are means.

* antioxidant significantly greater than control ($p<0.05$).

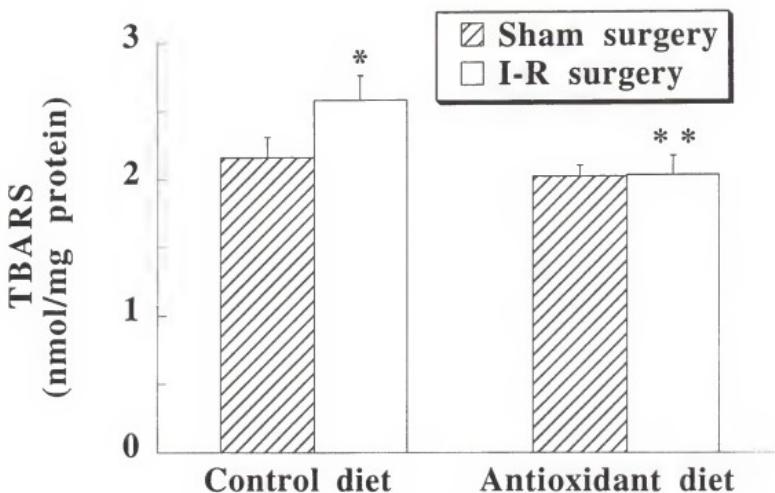


Figure 5. Comparison of TBARS concentrations in the myocardium of rats undergoing either I-R or sham surgery and consuming either a control diet or a supplemented diet.

Values are means (\pm SEM).

* significantly greater than sham surgery animals ($p < 0.05$).

** significantly less than control diet I-R surgery animals ($p < 0.05$).

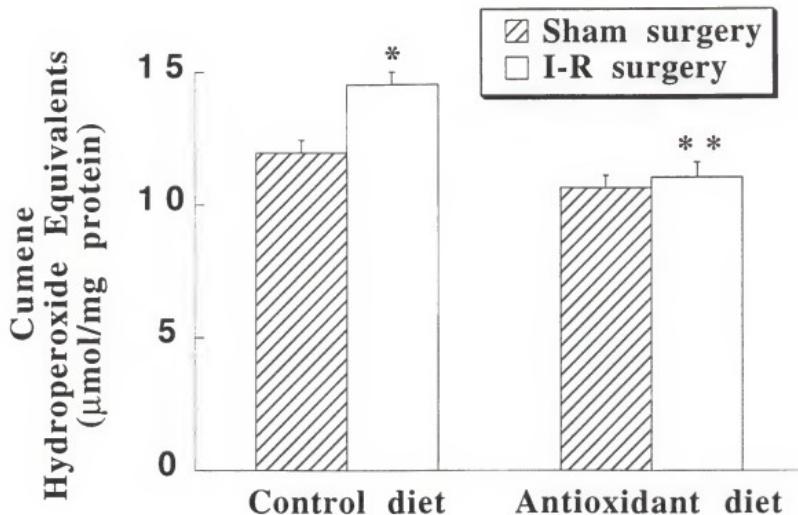


Figure 6. Comparison of hydroperoxide concentrations in the myocardium of rats undergoing either I-R or sham surgery and consuming either a control diet or a supplemented diet.

Values are means (\pm SEM).

* significantly greater than sham surgery animals ($p<0.05$).

** significantly less than control diet I-R surgery animals ($p<0.05$).

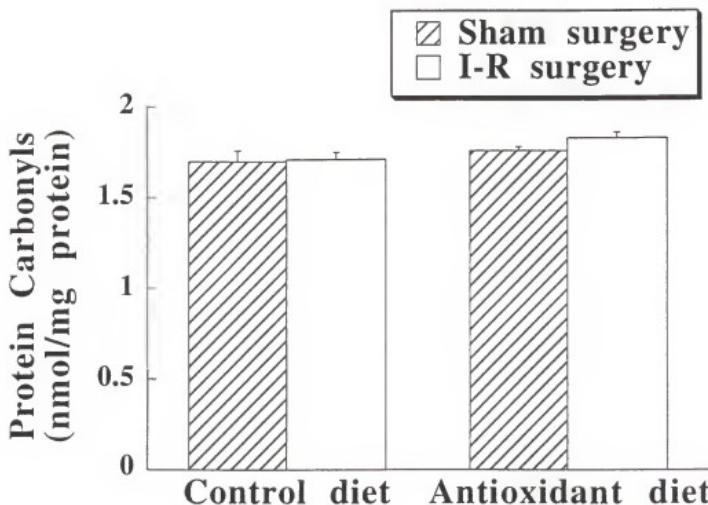


Figure 7. Measurement of protein carbonyls in the myocardium of aged rats consuming either a control diet or an antioxidant supplemented diet. Values are means (\pm SEM). No significant differences existed between groups ($p>0.05$).

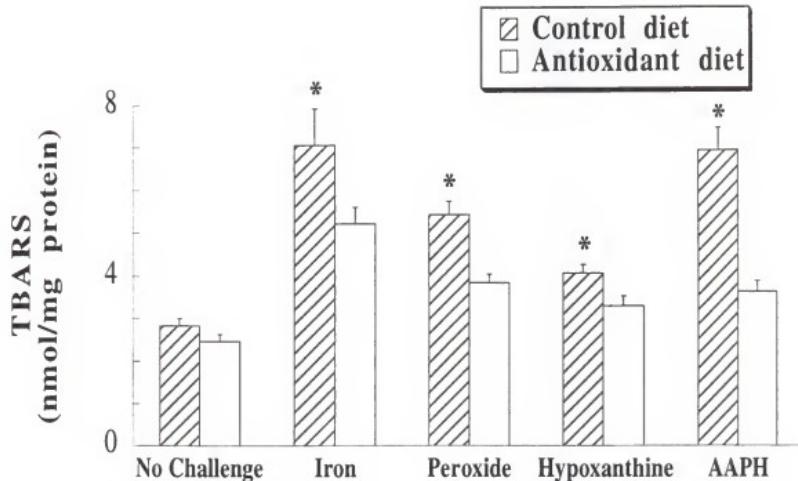


Figure 8. Lipid peroxidation (TBARS assay) in heart homogenates from either control diet or antioxidant supplemented animals subjected to four different *in vitro* oxidative challenges (aqueous phase).

Values are means (\pm SEM)

* significantly greater than antioxidant supplemented animals exposed to the same treatment ($p < 0.05$).

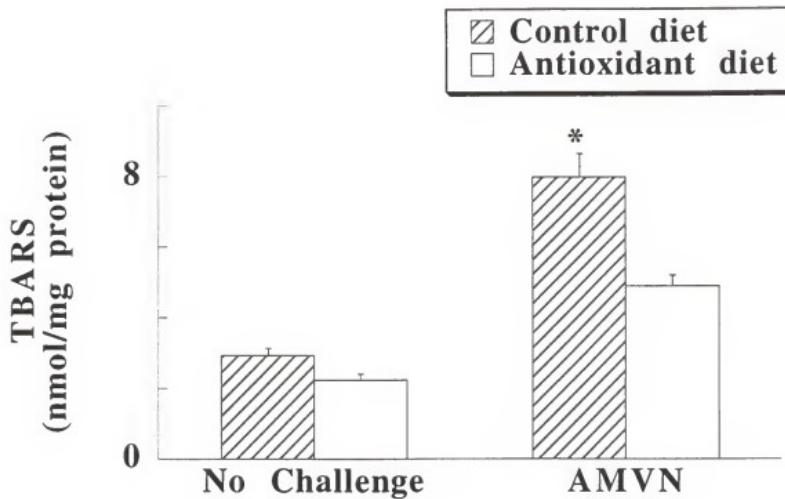


Figure 9. Lipid peroxidation (TBARS assay) in heart homogenates from either control diet or antioxidant supplemented animals subjected to an *in vitro* oxidative challenge (lipid phase).

Values are means (\pm SEM).

* significantly greater than antioxidant supplemented animals exposed to the same treatment ($p < 0.05$).

CHAPTER 5 DISCUSSION

Overview of Principal Findings

This is the first experiment to examine the effects of VE and LA supplementation on cardiac performance and biochemistry during *in vivo* I-R in aged rats. A major finding of this study was that the antioxidant supplementation improved cardiac performance during post-ischemia reperfusion. In addition, it was hypothesized that the dietary combination of VE and LA would reduce myocardial lipid peroxidation from the *in vivo* I-R insult. This hypothesis was supported, as both markers of lipid peroxidation significantly ($p<0.05$) decreased after I-R in antioxidant supplemented animals compared to animals on a control diet. Further, *in vitro* experiments demonstrated that this dietary antioxidant combination protected the myocardium against lipid peroxidation induced by five different radical generating systems. Collectively, these data indicate that dietary supplementation with VE and LA results in a heart that resists lipid peroxidation by a variety of ROS and that this protection is associated with improved contractile recovery during reperfusion following myocardial ischemia.

Myocardial Vitamin E Levels

Based on reports {28, 63} of a time-dependent increase in cardiac VE levels during VE supplementation, animals were supplemented with VE and LA for 14 weeks. Our data revealed that this feeding protocol resulted in a significant (100%) increase in VE

concentration in the myocardium of aged rats. This diet-induced increase in myocardial VE concentration was not as great as previously reported in younger animals on an identical diet {28}. Several possibilities could explain this observation. First, intestinal absorption of VE could decrease with aging. However, Hollander and Dadufalxa {63} reported an age-related increase in the total amount of VE absorbed; these investigators concluded that this was an adaptation designed to increase the systemic availability of VE with aging. A second possibility is that VE is metabolized in the circulation prior to uptake by tissues. Nonetheless, this does not appear to occur as Machlin and Gabriel {83} reported no change in the urinary concentration of the major metabolite of alpha-tocopherol. A third possibility is that there is a decreased tissue uptake of the vitamin. Although the exact mechanism of VE uptake has not been elucidated, it is believed to occur by passive diffusion {15}. It follows that the rate of diffusion is dependent upon the VE concentration gradient between the plasma and the lipid phase of the cell membrane as well as the availability of lipid carriers {15}. However in experiments using isolated myocardial cells, it was reported that the uptake of VE does not change with aging {50}. A final possibility is that the cellular VE requirement increases with aging as it is required to quench more ROS than in younger animals.

In support of this notion, Chen et al. {25} reported that older mice required significantly more dietary VE than younger mice to prevent lipid peroxidation in liver homogenates. In addition, Ames et al. {5} used erythrocyte hemolysis as a marker of VE requirement and found that in old rats the increase in red cells lysis can be limited by increasing the intake of VE. Furthermore, although controversial, many investigators have shown a decrease in antioxidant enzyme activity in aged animals {23, 30, 113, 118}. This would increase the burden on other antioxidants such as VE and may help explain why the requirement of VE changes with age.

Myocardial Lipoate Concentrations

Myocardial lipoate content was not significantly influenced by dietary supplementation with LA. This finding is in agreement with previous work from our laboratory using the same dietary regimen in younger animals [28]. To date, only one other known group of investigators has fed LA to rodents and measured tissue levels [97]. These investigators employed a similar feeding protocol in mice and reported an increase in myocardial lipoate content. Possible explanations for the different results between this published report and the current study include: 1) species differences in the myocardial metabolism of lipoate; and/or 2) the incorporation of lipoate into proteins. Species differences between mice and rats might result in lipoate being quickly metabolized in the myocardium of rats via beta oxidation to short chain derivatives [119]. Although these metabolites also possess antioxidant capabilities [14, 125], we are unable to assess their contribution to the myocardial protection from lipid peroxidation observed during our experiments.

A second possibility is that myocardial lipoate content differed between groups but was undetectable due to the incorporation of lipoate into proteins. Indeed, lipoate can be incorporated into at least five proteins where it is covalently linked to a lysyl residue [92]. Our lipoate assay is capable of detecting free lipoate only.

Antioxidant Supplementation Improves Cardiac Performance During Reperfusion

A major finding of this study is that dietary supplementation with VE and LA improved contractile performance during myocardial reperfusion following ischemia. The extent of contractile dysfunction is dependent on the period of ischemia with greater than five minutes of ischemia resulting in myocardial stunning and a subsequent decreased contractile performance. Upon reoxygenation of hypoxic heart tissue, contractile

performance generally remains depressed for several days. The cause of this reperfusion-induced contractile dysfunction is believed to be due, at least in part, to an increased production of ROS during reoxygenation of the hypoxic tissue. The ROS have been shown to participate in degenerative cellular processes such as membrane lipid peroxidation and protein oxidation {26, 55, 60, 106, 112}. Damage to the sarcoplasmic reticulum membrane and calcium transport proteins may result in dissipation of the important transsarcolemmal calcium gradient and an increase in cytosolic calcium concentration. This causes a sustained contractile activation resulting in hypercontracture, distortion of the myocardial cytoskeleton and diminished contractile performance{43}. The present study suggests that this chain of deteriorating events might be attenuated by pre-loading the myocardium with nutritional antioxidants that decrease the ROS-induced hypercontracture by decreasing the amount of lipid peroxidation.

Again, this is the first study to demonstrate *in vivo* myocardial protection from I-R injury using the combination of VE and LA. Our findings are in agreement with previous reports of myocardial benefits *in vitro* using VE alone {9, 38, 41, 42, 46, 52, 74, 85, 91, 101} or a combination of VE and LA {58, 59}. In contrast, the current data are contradictory to a previous study in our lab using young animals {28}. At least two possibilities might explain these discrepancies. Firstly, the cardioprotection observed in the current study may be due to a diminished antioxidant capacity in the older animals compared to young. Our data support this hypothesis as SOD activity was lower in control animals following I-R compared to antioxidant supplemented animals.

A second possibility is that gender or strain differences may exist in the response of the animals to I-R. The previous study from our lab used female Sprague Dawley rats, whereas the present study used male Fisher 344 rats. In this regard, it has been reported that enzymatic antioxidant defenses, specifically SOD and CAT, varied with gender and strain in rats {105}. Furthermore, the female sex hormone estrogen and its metabolites

have been shown to have antioxidant capability {129}. Therefore, collectively, it appears that both gender and strain differences may contribute to our divergent findings.

Antioxidant Supplementation Reduces I-R Induced Myocardial Lipid Peroxidation

Lipid peroxidation is one of the most damaging process that occurs to the myocardium during I-R {115}. The oxidative modification of lipids results in alterations in the fluidity and permeability of membranes. It has been reported that lipid peroxidation of the sarcoplasmic reticulum occurs during I-R {107} which would result in altered calcium handling and subsequent contractile dysfunction {55}. In the present study, two measures of lipid peroxidation were used to determine if dietary supplementation reduced left ventricular lipid damage following I-R. Compared to sham animals, I-R increased myocardial lipid peroxidation in control diet animals. In contrast, compared to sham, I-R did not increase myocardial MDA and CHE levels in animals supplemented with VE and LA.

These findings are in agreement with studies that have reported decreased *in vitro* I-R induced lipid peroxidation with pre-feeding of VE alone {9, 38, 41, 42, 46, 52, 74, 85, 91, 101} or in combination with LA {58, 59}. However, the current study is the first investigation to support the notion that this dietary antioxidant regimen reduces I-R induced lipid peroxidation in the heart under physiological conditions.

In Vitro Oxidative Challenges

In an effort to elucidate the specific ROS involved in the antioxidant-induced protection from lipid peroxidation, a series of experiments were conducted that oxidatively challenged heart homogenates from both control diet and supplemented sham surgery animals. It was

hypothesized that heart homogenates from aging animals fed the antioxidant diet would quench superoxide radicals, hydroxyl radicals, hydrogen peroxide and peroxy radicals generated *in vitro*. Our data support this postulate. Indeed, after exposure to five ROS generating systems, lower levels of TBARS were detected in heart homogenates from supplemented animals compared to control diet animals.

Previous work has shown that heart homogenates from animals supplemented with VE are protected against AMVN and AAPH induced accumulation of TBARS, but not superoxide radicals, hydroxyl radicals or hydrogen peroxide {80, 86}. Further, the addition of LA and DHLA to heart homogenates results in protection against superoxide radicals, the hydroxyl radicals and hydrogen peroxide {69}. Therefore, the finding that the nutritional combination of the two antioxidants results in a myocardium that is protected against all five ROS generating systems is in general agreement with previous research. Collectively, the data support the notion that combining aqueous and lipid phase antioxidants is therapeutically more beneficial.

Myocardial I-R and Protein Oxidation

Surprisingly, myocardial levels of protein carbonyls were not significantly greater in animals exposed to I-R surgery compared to the sham surgery animals. This indicates that at the time of tissue harvesting, the markers of protein oxidation had not been effected by the I-R insult. This finding disagrees with our hypothesis as well as other studies which have reported increased myocardial protein carbonyl concentrations following I-R in young animals {100, 102}. At least two possibilities may explain the divergent findings. First, this current study is the only known investigation that has examined the effects of myocardial I-R on protein carbonyl accumulation in the hearts of aged animals. Although it is known that myocardial protein carbonyl accumulation increases with aging {102}, it is possible that the increased resting levels in aging may mask any further increase when

tissue is subjected to an oxidative stress. This is a testable hypothesis and warrants further investigation.

A second possible explanation is that in the I-R animals, protein carbonyl levels may have peaked before the tissues were harvested. This notion is supported by Poston and Parenteau {100} who found that carbonyl levels rose during myocardial ischemia and were over four times the initial values at five minutes of reperfusion but, with continued reperfusion, declined to only 150% of initial values at 15 minutes of reperfusion. An increased level of glutathione in aged animals {66} might explain a decrease in protein carbonyl levels over time during reperfusion. Glutathione has been described as the most important cytosolic antioxidant {105} and this localization would allow it to directly reduce protein carbonyl groups.

Effects of Antioxidant Supplementation and I-R on Myocardial Antioxidant Enzymes

The effect of the antioxidant supplementation on activities of myocardial enzymes important in protection against oxidative stress was also investigated. Two interesting observations warrant discussion. First, activity increased in antioxidant supplemented animals in both the sham and I-R groups. This finding agrees with previous data from our laboratory {28} as well as from other investigators {111}. The explanation for the increased GPX activity with VE supplementation could be due to an increase in cellular selenium concentrations that may accompany VE supplementation {111}. Selenium is a cofactor for GPX and higher cellular levels of vitamin E has been shown to stimulate increased expression of the enzyme {111}.

The second interesting finding was a significantly greater total SOD activity in the myocardium of antioxidant supplemented animals exposed to I-R surgery compared to control diet I-R surgery animals. Also, myocardial total SOD activity was lower in the

control diet I-R surgery animals compared to control diet sham surgery animals. These differences in myocardial SOD activity were due to changes in the activity of the manganese isoform. The decreased activity of this mitochondrial isoform of SOD in aged animals exposed to myocardial I-R may be the result of down regulation due to an increased production or decreased removal of hydrogen peroxide. Indeed, it has been demonstrated that hydrogen peroxide is a negative allosteric modifier of mnSOD activity {19}. In the supplemented animals, the presence of additional antioxidants such as LA, DHLA or their metabolites, all of which have been shown to quench hydrogen peroxide, may protect mnSOD from down regulation. These findings also help explain why the antioxidant supplementation was more effective in the aged animals than in a previous study we conducted with young animals {28}

Critique of the Model

The male Fisher 344 rat was chosen as experimental model because: 1) the nature of these invasive experiments precludes the use of human subjects; 2) this rat is highly inbred and does not display large inter-animal variation in coronary collateral circulation {6}; and 3) the rat is widely accepted model for the study of dietary antioxidant interventions and an accepted model for aging research {9, 34, 70, 91}.

The decision to use 10,000 IU of VE and 1.65 g of LA per kg diet was based on previous work that has shown that these dosages of VE {52, 53} and LA {92} have given beneficial results. Also, this dietary dosage of VE results in serum tocopherol levels in the rat of 3 mg/dl {76} which is similar to values obtained in humans when large doses (600 IU/day) of alpha tocopherol are consumed {119}.

The surgical procedure used in these experiments has been used successfully by numerous investigators and has been shown to result in both myocardial ischemia and reperfusion {12, 27}. However, it is possible that this type of experimental surgery

could result in inter-animal differences in either the magnitude of ischemia or reperfusion. Nonetheless, we believe that these differences are clinically relevant and probably better reflect the types of I-R insults that occur in humans.

The decision not to include additional experimental groups that consumed only VE or LA was based on the data from Haramaki et al. {52}. These investigators reported that it was the combination of the two antioxidants that provided protection and fewer benefits were observed when they were used individually.

Summary and Conclusions

These experiments examined the effects of dietary supplementation with VE and LA on myocardial physiological and biochemical responses during *in vivo* I-R in the aged rat. The dietary regimen resulted in a significant increase in myocardial VE content following 14 weeks of supplementation. Dietary supplementation with these antioxidants improved cardiac contractile performance during reperfusion following ischemia. This improvement in recovery from ischemia appears to be due to the supplemented hearts being protected from a wide range of reactive oxygen species resulting in reduced lipid peroxidation. These results indicate that this combination of antioxidant supplements may provide therapeutic prevention against myocardial ischemia-reperfusion injury in the clinical setting.

Potential Future Research

The results of our experiments raise several interesting questions that warrant further research. For example, our finding that dietary supplementation of VE in old animals resulted in lower myocardial levels of VE compared to our previous study with young

adult animals. The mechanism to explain this finding is unclear and is worthy of investigation.

Another interesting observation in these experiments was the finding that our antioxidant supplementation protocol resulted in reduced down-regulation of myocardial SOD activity following I-R. The mechanism responsible for this finding is unknown and should be investigated. Finally the results of these experiments clearly indicate that antioxidant supplementation with both VE and LA provides myocardial protection against an I-R insult in aged rats. These findings are important and suggest that dietary antioxidant supplementation may also protect humans against an I-R insult. Therefore, there is a clear need for future clinical trials using nutritional antioxidants in a variety of populations that are at risk for I-R injury.

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BIOGRAPHICAL SKETCH

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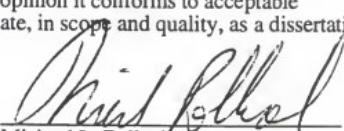
Scott K. Powers, Chair
Professor of Exercise and Sport Sciences

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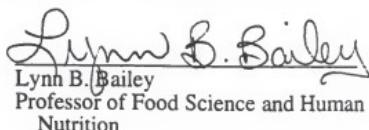
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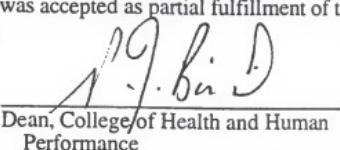
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April, 1998



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